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Gerronema nemorale: first report of the genus and species from Pakistan

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Abstract—Basidiomata of a *Gerronema* species, collected in Murree, Punjab, Pakistan, have been confirmed as *G. nemorale* by morphological and ITS1-5.8S-ITS2 rDNA sequence analyses. This is the first report of the genus *Gerronema* from Pakistan, and the first record of *G. nemorale* for the South Asian region.

Key words—*Agaricales*, *Agaricomycetes*, *Basidiomycota*, lignicolous agarics, sarcodimitic

Introduction

Gerronema Singer (*Agaricales*, *Basidiomycota*) was created by Singer (1951) to accommodate three omphalinoid–clitocyboid species from South America. The addition of several additional species (Singer 1961, 1964, 1975, 1986) caused it to be regarded as heterogeneous by many other authors. Molecular phylogenetic analysis by Lutzoni (1997) using combined nrITS and nrLSU datasets suggested that *Gerronema* was polyphyletic. Redhead (1986) restricted *Gerronema* to species with sarcodimitic tissues; including the type, *G. melanomphax* Singer. Norvell & al. (1994) limited *Gerronema* to species with a lignicolous habitat, thin-walled basidiospores, and sarcodimitic tissues. *Gerronema* in this restricted sense is monophyletic and falls in the /hydropoid clade (Moncalvo & al. 2002; Antonín & al. 2008).

Gerronema as recently accepted is a genus of small- to medium-sized white-spored lignicolous agarics with decurrent gills, inamyloid thin-walled basidiospores, and sarcodimitic tissues (Norvell & al. 1994). The genus has a primarily tropical distribution but is also found in Europe and eastern North America where it produces basidiomata during hot, muggy summer weather (Norvell & al. 1994; Redhead & al. 2002). The genus is represented by 125 species worldwide (www.speciesfungorum.org, accessed on 27 December 2020).

During a routine macrofungal survey, specimens were found which resembled *Gerronema*. Detailed morphological and ITS sequence analyses were performed to identify the specimens to species.

Material & methods

Sampling site

Murree, an important Himalayan hill station (33.35°N 73.27°E, 2290 m asl) lies 50 km northeast of Islamabad (the capital of Pakistan) in the Himalayan foothills (murreehill.com/introduction.html). The subtropical monsoon climate has an annual precipitation of c. 1800 mm, with the maximum precipitation occurring in July and August (World Weather Information Service 2020).

Pinus roxburghii Sarg. dominates the forest regions near Murree, with *P. wallichiana* A.B. Jacks. found at the higher elevations. *Abies pindrow* (Royle ex D. Don) Royle, *Cedrus deodara* (Roxb. ex D. Don) G. Don, and *Picea smithiana* (Wall.) Boiss., are scattered sparingly throughout the Murree forests (Hameed & al. 2012). *Aesculus indica* (Wall. ex Cambess.) Hook., *Quercus dilatata* Royle, and *Q. oblongata* D. Don [= *Q. incana* Roxb.] are among the deciduous broadleaf trees in the upper regions (Beg 1975). Shrubby species like *Carissa opaca* Stapf ex Haines, *Dodonaea viscosa* (L.) Jacq., *Justicia adhatoda* L., and *Myrsine africana* L., are also present (Hussain & Illahi 1991).

The soils are classified as Entisols and Inceptisols, with more acid soils predominating above 2000 m. The soils are typically loamy and shallow, and their development has been restricted due to the steepness of the slopes (Soil Survey of Pakistan 1987).

Collection & morphological characterization

Basidiomata were collected, tagged, photographed, and characterized morphologically. Colors were designated following the Munsell Soil Color Charts (1994). The specimens were hot-air dried for preservation. Microscopic features, measurements, and drawings were made from tissues rehydrated and crush-mounted in 5% KOH and stained with Congo red (1% aqueous) or in Melzer's reagent. Basidiospores, basidia, cystidia, and pileal and stipitipellis elements were examined with an Olympus CH 30RF 200 compound microscope equipped with a digital camera; measurements were recorded using ScopeImage 9.0(x5) version 1.00. For basidiospores, numbers follow the formula [n/m/p] = n basidiospores, m specimens, p collections. Basidiospore dimensions follow (a–)b–c(–d), where b–c contains at least 90% of the measured values with extreme values given in parentheses. The average length and width of basidiospores is represented by avl and avw. Quotient Q is used to symbolize the length/width ratio of individual spores; avQ represents the Q average of all basidiospores. The examined collections were deposited in the herbarium of the Department of Botany, University of the Punjab, Lahore, Pakistan (LAH).

DNA extraction and PCR amplification

Genomic DNA was extracted from 30–50 mg of dried basidioma in a 2% CTAB extraction buffer, following the Bruns (1995) protocol with some modifications. The nrDNA ITS region was amplified using ITS1F (forward) and ITS4 (reverse) primers (White & al. 1990). The polymerase chain reaction (PCR) was performed with the Extract-N-Amp PCR kit in 20 µl reaction volumes according to Gardes & Bruns (1993). The size of the PCR product was determined on a 1% agarose gel in a gel documentation system (UVtec, Cambridge, UK) using default settings. The PCR products were directly sequenced by the Beijing Genomics Institute in both directions using the same pair of amplification primers. The newly generated sequences were deposited in GenBank.

Sequence alignment, dataset assembly, and phylogenetic analysis

Newly generated *Gerronema* ITS sequences, along with those retrieved in a GenBank BLAST search were used for the phylogenetic analysis. The ITS sequence dataset comprised the Pakistani sequences, those of other *Gerronema* species selected based on their similarity indices, and other taxa that were used in previous phylogenetic analyses (Cooper 2014; Antonín & al. 2008; Latha & al. 2018). *Multiclavula corynoides* (Peck) R.H. Petersen and *M. vernalis* (Schwein.) R.H. Petersen were selected as outgroup, following Antonín & al. (2008).

The dataset was aligned using MAFFT (Katoh & Standley 2013). DNA sequences were trimmed with the conserved motifs 5'-(...GAT)CATTA- and -GACCT(CAAA...)-3' with the program BioEdit sequence alignment editor v.7.2.5.0 (Alzohairy 2011). MEGA 7 was used to select the best phylogenetic model, (Kumar & al. 2016). Bootstrap analysis was performed with 1000 replications. A Maximum Likelihood (ML) phylogenetic tree was generated using MEGA 7 with default settings (Kumar & al. 2016) under the K2 + G model (Nei & Kumar 2000).

Phylogenetic results

A megablast search of the GenBank nucleotide database using the nrITS (717–724 bp) Pakistani *Gerronema* sequences showed three *Gerronema nemorale* sequences from the Republic of Korea (EU883592–EU883594) with 99.5% similarity and a single sequence from China (LT716047) with 99.4% similarity. Complete alignment of the ITS region comprised 926 positions, including gaps. The analysis involved 42 nucleotide sequences. Models with the lowest BIC scores (Bayesian Information Criterion) were considered to best describe the substitution pattern. All positions containing gaps and missing

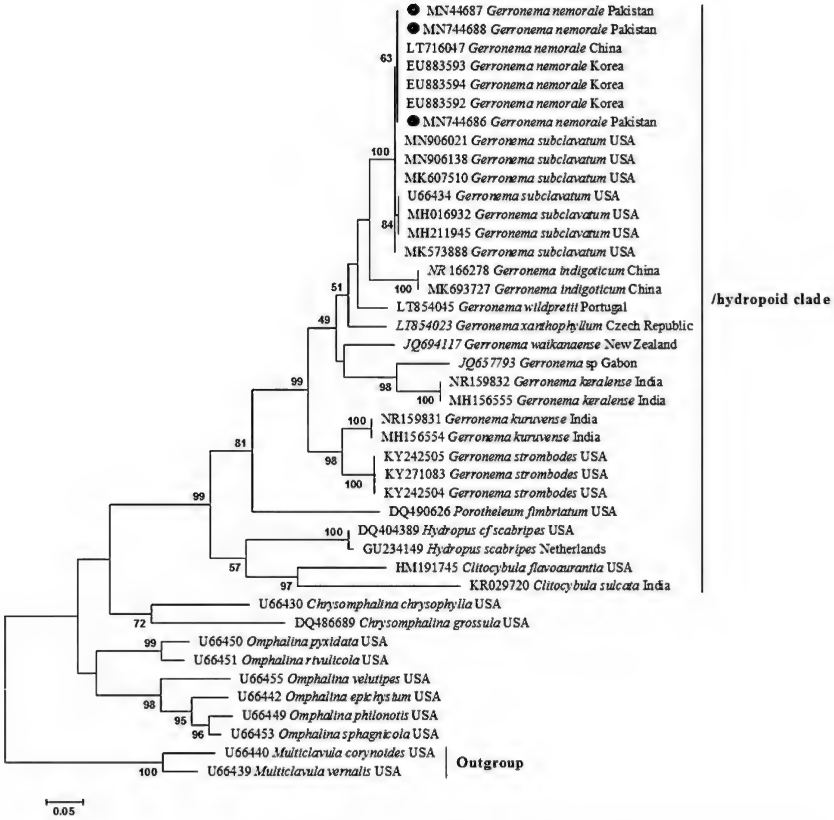
● MN44687 Gerronema nemorale Pakistan
● MN744688 Gerronema nemorale Pakistan
LT716047 Gerronema nemorale China
EU883593 Gerronema nemorale Korea
EU883594 Gerronema nemorale Korea
EU883592 Gerronema nemorale Korea
● MN744686 Gerronema nemorale Pakistan
MN906021 Gerronema subclavatum USA
MN906138 Gerronema subclavatum USA
MK607510 Gerronema subclavatum USA
U66434 Gerronema subclavatum USA
MH016932 Gerronema subclavatum USA
MH211945 Gerronema subclavatum USA
MK573888 Gerronema subclavatum USA
NR 166278 Gerronema indigoticum China
MK693727 Gerronema indigoticum China
LT854045 Gerronema wildpretii Portugal
LT854023 Gerronema xanthophyllum Czech Republic
JQ694117 Gerronema waikanaense New Zealand
JQ657793 Gerronema sp Gabon
NR159832 Gerronema keralense India
MH156555 Gerronema keralense India
NR159831 Gerronema kuruvense India
MH156554 Gerronema kuruvense India
KY242505 Gerronema strombodes USA
KY271083 Gerronema strombodes USA
KY242504 Gerronema strombodes USA
DQ490626 Porotheleum fimbriatum USA
DQ404389 Hydropus cf scabripes USA
GU234149 Hydropus scabripes Netherlands
HM191745 Clitocybula flavoaurantia USA
KR029720 Clitocybula sulcata India
U66430 Chrysomphalina chrysophylla USA
DQ486689 Chrysomphalina grossula USA
U66450 Omphalina pyxidata USA
U66451 Omphalina rivulicola USA
U66455 Omphalina velutipes USA
U66442 Omphalina epichysium USA
U66449 Omphalina philonotis USA
U66453 Omphalina sphagnicola USA
U66440 Multiclavula corynoides USA
U66439 Multiclavula vernalis USA
/hydropoid clade
Outgroup
63
100
84
100
51
49
99
98
100
100
81
98
100
99
100
57
97
72
99
98
95
96
100
0.05

Fig. 1. Molecular phylogenetic analysis by Maximum Likelihood; nrITS sequence-based ML phylogram depicting the placement of *G. nemorale* within *Gerronema*. Values at nodes indicate bootstrap support. Scale bar indicates the number of substitutions per site. Newly generated sequences are marked with ●.

data were eliminated during model estimation. K2 + G Model showed the following base frequencies A (0.250), C (0.250), G (0.250), T (0.250) and a gamma distribution shape parameter of 0.35. The nucleotide substitution rates estimated according to this model were A/G = 0.178, A/T = 0.036, C/G = 0.036, C/T = 0.178, and G/T = 0.036.

The ITS phylogenetic tree (Fig. 1) generated from the ML analysis nested the sequences in the /hydropoid clade with 99% bootstrap support. All *Gerronema nemorale* sequences and our new Pakistani sequences clustered together with strong bootstrap support (99%).

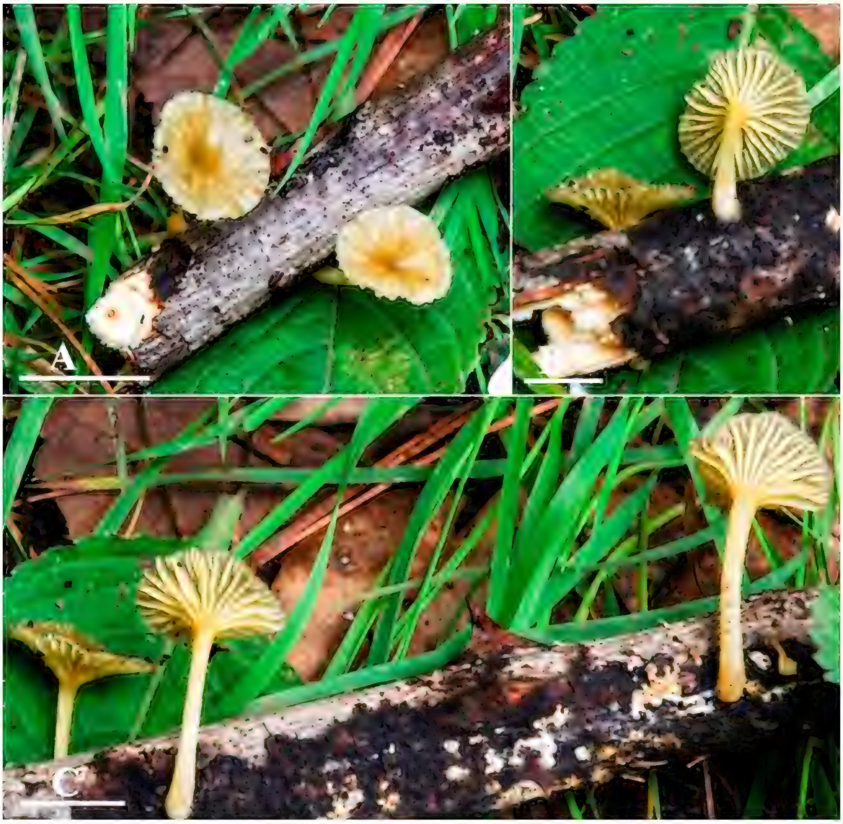

Fig. 2. *Gerronema nemorale.*
Basidiomata: A. FA249 (MN744686); B. FA236 (MN44687); C. FA239 (MN744688).
Scale bars = 1 cm.

Taxonomy

Gerronema nemorale Har. Takah., Mycoscience 41(1): 16 (2000) Figs 2–3

Basidiomata solitary or grouped. Pileus 10–13 mm diam, convex with umbilicate centre, margin inflexed, uplifted and wavy on maturity, innately finely radially fibrillose to striate-rugulose except for the centre, yellow to brown when young (2.5Y6/8–7/8), finally dull yellow (2.5Y8/4). Lamellae arcuate-decurrent, subdistant, narrow, thin, pale yellow (2.5Y8/4); edges fimbriate, concolorous, finely pubescent. Context hollow in stipe, yellowish (2.5Y8/8) in pileus. Stipe 20–34 × 1–2 mm, cylindrical, broader at apex, subbulbous at base, straight, central or eccentric, slender, firm, hollow, olive yellow to pale yellow (2.5Y6/8–8/4), scabrous all over. SPORE PRINT white. ODOR and TASTE not distinctive.

BASIDIOSPORES [60/8/3] (7.5–)8–9.7(–10) × (4.5–)5–5.9(–6) µm, avl × avw = 8.7 × 5.4 µm, Q = (1.50)1.60–1.64(–1.66), avQ = 1.6, ellipsoid to broadly ellipsoid, smooth, inamyloid, thin-walled, hyaline. BASIDIA 26–37 × 5–7.2 µm, clavate, with 4 sterigmata. BASIDIOLES 25–34 × 5–7 µm, clavate or cylindrical. CHEILOCYSTIDIA 26–51 × 4–9 µm, abundant, variable in shape (clavate, subcylindrical, subutriform, or subfusoid), smooth, colorless or with pale yellow contents, thin-walled. PLEUROCYSTIDIA absent.

PILEITRAMA HYPHAE cylindrical, sarcodimitic (a dual hyphal system with two types of generative hyphae: wide vessel hyphae that are non-septate, fusoid, and thick-walled and narrow flexuous hyphae (sometimes called binding hyphae) that are thin-walled, sinuous, and branched. PILEIPELLIS a cutis of thin-walled hyphae, 2–5 µm wide, cylindric, smooth, colorless or with pale yellow contents; terminal cells swollen (clavate), 2–9 µm wide, true pileocystidia not observed. STIPITIPELLIS a cutis of parallel hyphae, 2–10 µm wide, cylindric, smooth, colorless or with pale yellow contents, thin- to thick-walled; CAULOCYSTIDIA (terminal cells) 22–63 × 5–10 µm, irregular, cylindrical or clavate, often capitate, with contents yellowish in KOH; Stipititrama sarcodimitic (possessing a dual hyphal system composed of two types of generative hyphae) CLAMP CONNECTIONS present in all tissues.

SPECIMENS EXAMINED—**PAKISTAN**, **PUNJAB**, Murree Hills, 2290 m a.s.l. solitary to caespitose, on dead fallen twigs on forest floor in mixed forests of *Abies pindrow*, *Cedrus deodara*, *Pinus roxburghii*, *P. wallichiana*, *Quercus dilatata*, *Q. oblongata*, 14 August 2018, Fauzia Aqdus FA249 (LAH36351, GenBank MN744686); FA236 (LAH36370, GenBank MN44687); FA239 (LAH36372, GenBank MN744688).

DISTRIBUTION—*Gerronema nemorale* has previously been recorded from Japan, Republic of Korea, and China.

Discussion

Gerronema nemorale was originally described from Japan (Takahashi 2000) and subsequently reported from Korea (Antonín & al. 2008); its presence in China is suggested by an unpublished sequence LT716047 in GenBank. Our collections are the first record of the species from South Asia and the first record of the genus from Pakistan.

The Pakistani basidiomata exhibit the omphalinoid appearance, decurrent lamellae, thin-walled smooth basidiospores, sarcodimitic tramal tissues, abundant cystidia on the stipe, and lignicolous habitat that led us to *Gerronema* (Singer 1970, 1986).

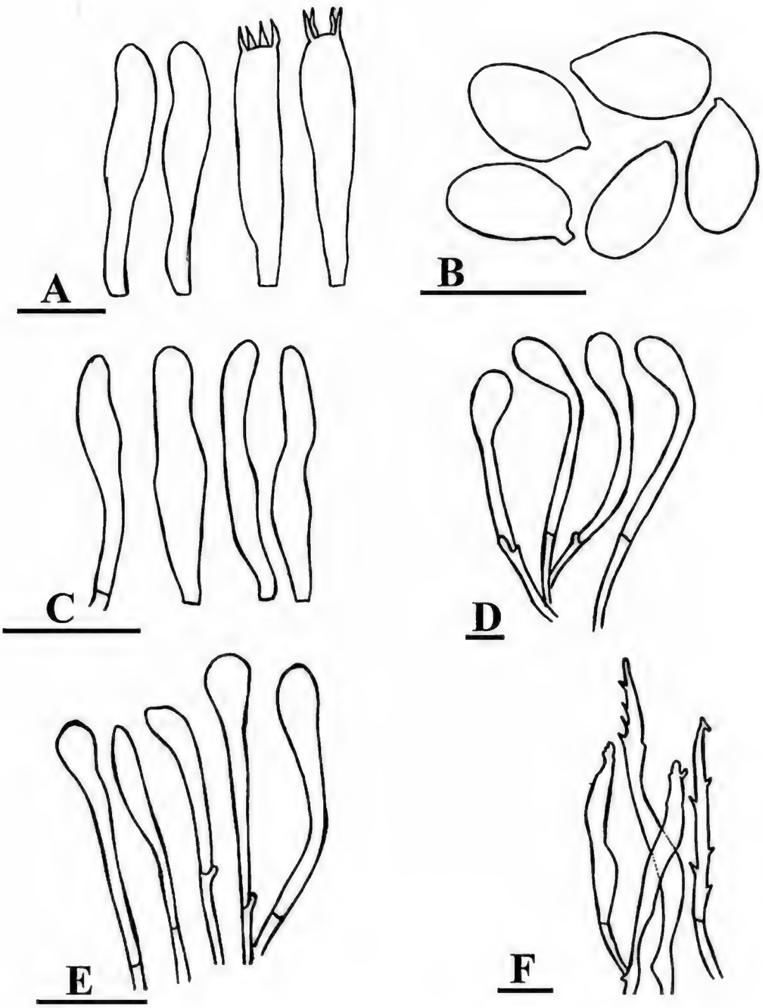

Fig. 3. *Gerronema nemorale* (FA249; MN744686):
A. Basidia and basidioles; B. Basidiospores; C. Cheilocystidia;
D. Pileipellis; E. Stipitipellis; F. Tramal hyphae. Scale bars = 10 µm.

Gerronema subclavatum (Peck) Singer ex Redhead (described by Bigelow 1970 as *Omphalina subclavata*) from North America resembles *G. nemorale* in its plano-convex pileus with a depressed center and incurved margin, fibrillose pileus surface, decurrent lamellae, smooth inamyloid ellipsoid and similarly sized (6–10 × 4–5.5 µm) basidiospores, absence of pleurocystidia, and lignicolous habitat. However, *G. subclavatum* is distinguished by its broader (3–25 mm) pileus that is subhygrophanous, infundibuliform in age, and more dully colored (with olive to brownish olive hues); slightly fungoid, disagreeable, or bitter taste; larger (27–40 × 5.5–8.5(–10) µm) basidia that are rarely 2-spored; occasional cheilocystidia; very scattered habit; and versiform pileocystidia (Bigelow 1970). A pair-wise alignment comparing *G. nemorale* ITS sequences with *G. subclavatum* (MK607510) showed 99.2% similarity. Phylogenetic studies showed a close relationship between both taxa despite the morphological differences. We were unable to examine the type specimens, and more research needs to be done in the future to resolve this issue.

Gerronema strombodes (Berk. & Mont.) Singer (FJ596789) showed only 90.4% similarity with *G. nemorale* (FA249) in a BLASTn search using the ITS sequence (717 bp). A species originally described from USA (Singer 1961), *G. strombodes* is morphologically similar to *G. nemorale* in its fibrillose pileus with depressed center, subdistant lamellae, ellipsoid basidiospores, and absence of pleurocystidia and pileocystidia. However, *G. strombodes* is distinguished from the Pakistani collection by its larger basidiomata, grayish white pileus, longitudinally striped stipe that is slightly pruinose at the apex, 1–4-spored basidia, and absence of caulocystidia (Singer 1961). *Gerronema strombodes* was removed from *Chrysomphalina* and retained in *Gerronema* based on its sarcodimitic tissues; and the new combination *G. xanthophyllum* (Bres.) Norvell & al. was proposed for a vicariant European taxon that had been confused with *G. strombodes* (Norvell & al. 1994). The name *G. xanthophyllum* [≡ *Clitocybe xanthophylla* Bres., = *Omphalia hypoxantha* Bres.] was selected based on examination of original material that most closely matched the European concept to which *G. strombodes* had been misapplied (Norvell & al. 1994).

Gerronema waikanaense (G. Stev.) J.A. Cooper (Cooper 2014) was originally described by Stevenson (1963; as *Hygrophorus waikanaensis*). This species shares with *G. nemorale* decurrent lamellae, 4-spored basidia, inamyloid basidiospores, absence of pleurocystidia and pileocystidia, and cylindrical caulocystidia. However, *G. waikanaense* is distinguished by its broader (10–30 mm) basidiomata, the minutely fibrillose pileus surface, and absence of cheilocystidia (Cooper 2014). A pair-wise alignment comparing the ITS sequences of *G. nemorale* and *G. waikanaense* (JQ6941171) showed only 87.5% similarity.

Gerronema kuruvense K.P.D. Latha & Manim. described from Kerala State, India (Latha & al. 2018) resembles *G. nemorale* in its pileus with a depressed center, subdistant lamellae, a stipe with a subbulbous base, absence of pleurocystidia, and the presence of caulocystidia. However, *G. kuruvense* is distinguished by its slightly smaller (≤4–11 mm diam.) pileus that is hygrophanous, 2-spored basidia, absence of cheilocystidia, and the presence of true pileocystidia (Latha & al. 2018). A pair-wise alignment comparing the ITS sequences of *G. nemorale* and *G. kuruvense* (NR120035) showed only 87.1% similarity.

Gerronema indigoticum T. Bau & L.N. Liu described from Guangxi Province, China (Liu & al. 2019), resembles *G. nemorale* in its umbilicate pileus with an inflexed (eventually uplifted) margin that is innately finely radially fibrillose to striate, decurrent lamellae, a broadly based stipe, absence of pleurocystidia and true pileocystidia, and the presence of cheilocystidia and caulocystidia. However, *G. indigoticum* is distinguished by its blue to blue-green basidiomata and slightly larger (≤9–16 mm diam.) pileus, smaller (6.8–8.8 × 3.7–4.4 µm) basidiospores, 2–4-spored basidia, and smaller but broader cheilocystidia (20–27 × 8–12 µm; Liu & al. 2019).

Finally, *G. nemorale* somewhat resembles *G. wildpretii* Bañares & al. from Canary Islands, Spain (Bañares & al. 2006), in its similar umbilicate pileus, subdistant lamellae, stipe with a broadened base, absence of pleurocystidia, and presence of cheilocystidia. *Gerronema wildpretii* is distinguished by its larger (25–70 mm diam.) hygrophanous pileus, 1–3-spored (rarely 4-spored) basidia, and true pileocystidia, and absence of caulocystidia (Bañares & al. 2006).

BLASTn search results and the ITS-based phylogenetic analysis supports *G. nemorale* among the other species of *Gerronema*. Our ITS phylogeny nests the Pakistani sequences in the /hydropoid clade with 100% bootstrap support together with *Hydropus* s. str., *Clitocybula*, and *Porotheleum fimbriatum* (Moncalvo & al. 2002). All *G. nemorale* sequences clustered together with strong bootstrap support.

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